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AUTHOR(S):

KUBOTA, Koji; KURODA, Hiroyuki; SAKAI, Fukumi

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Preliminary

Expression of Stilbene Synthase Gene in Japanese Red Pine *(Pinus densiflora)* Seedlings*[1]

Koji KUBOTA*[2,3], Hiroyuki KURODA*[2],
and Fukumi SAKAI*[2]

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Abstract——The present paper shows that stilbene synthase gene *(sts)* expresses with or without salicylate (SA) treatment in Japanese red pine seedlings, the chemical that is known as a key substance of angiosperms against diseases. The effect of SA was diversed in those seedlings, that may reflect the divergent response within the species.

Keywords: stilbene synthase gene, gene expression, stress responce, pine seedlings, *Pinus densiflora*

1. Introduction

The stilbene derivatives are typical extractives in pine heartwood and in the stressed tissues[1]. They show week but well-defined anti-fungal activities and are clearly demonstrated as a phytoalexin because the gene introduced into tobacco plants gave the plants resistant to fungal attack[2]. A few German groups have been studied for the stilbene biosynthesis in a pine, especially, in Scots pine *(Pinus sylvestris)*. For example, the pine seedlings express stilbene synthase gene *(sts)* with a stress, e.g., UV irradiation, fungal attack, and ozone exposure[3]. This gene is responsible for the direct formation of pinosylvin from three malonates and one cinnnamate[3,4] (Fig.1).

We employed Japanese red pine *(Pinus densiflora)* as a plant material. Pine trees in Japan have suffered severely from pine wilt diseases[5]. We are interested in *sts* expression of the red pine, because it is directly related to the phytoalexin formation, and may give an acquired resistance to the conifer.

Treatment with salicylic acid (SA) is able to mimic pathological stresses in many plants[6]. The stresses induce systemic acquired resistance or several pathogenesis-related (PR) proteins in a wide range of angiosperms[7]. Endogenous SA levels increase and several PR proteins are induced during the plant response to pathogen infection[8]. These are strong evidences that SA in angiosperm plays an important role in signal transduction pathway leading to the systemic acquired resistance. However, not all plant-pathogen

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*[2] Laboratory of Gene Expression.

*[3] Present address: Graduate School of Medicine, Kyoto Univ..

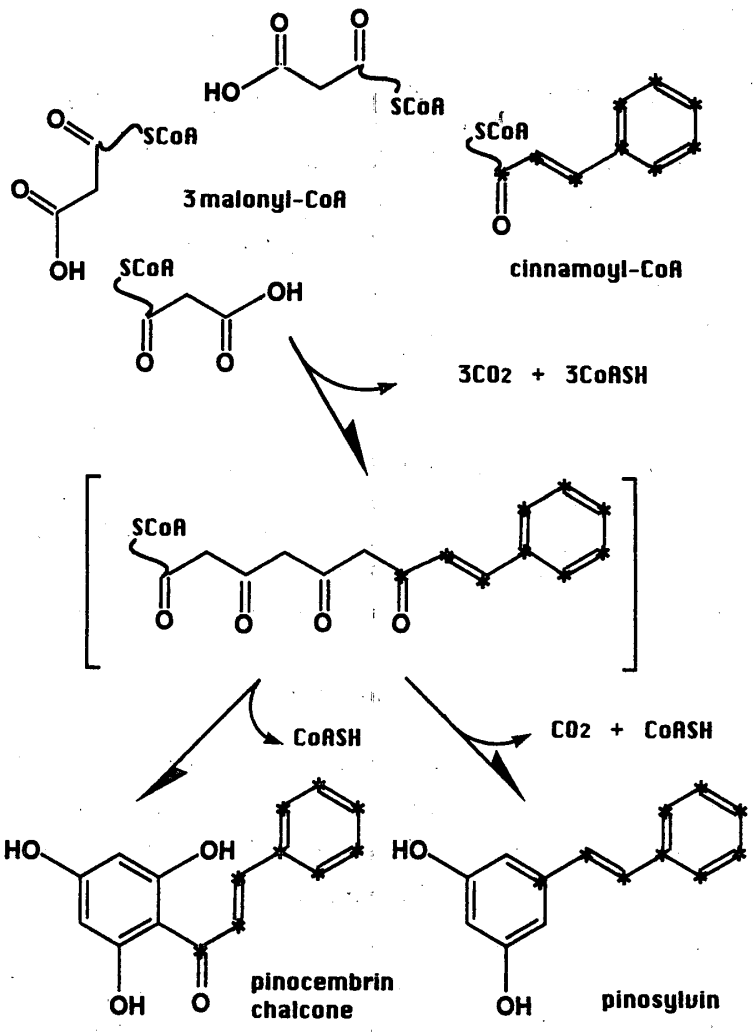

Fig. 1. Formation of a stilbene and a chalcone by the respective synthases. Both of the enzymes utilize same substrates, i.e., three malonates and a cinnamate to form tetraketide intermidiate, followed by ring formation with or without decarboxylation. Astarisks in the compounds show cinnamate carbon skeleton.

systems respond to SA, nor report on the effect of SA on gymnosperm.

This paper focused on *sts* if it may or may not express with the SA treatment in a gymnosperm, *Pinus densiflora*.

2. Materials and Methods

The seeds of Japanese red pine, *Pinus densiflora*, were purchased from a local market. The seedlings were grown on vermiculite under dark at 28°C after soaking the seeds with tap water for 2 days. At the 15 days after seeding, 10mM of sodium salicylate had sprayed over the seedlings that were further incubated for 0 to 32 hours under the same condition. One gram fresh weight of the sample harvested was powdered with liquid nitrogen in a mortar

and extracted with guanidium salt. After removal of cell debris and polysaccharide by passing through a RNeasy spin column (Qiagen), one microgram of total RNA was immediately reverse-transcribed according to the manufacturer's instruction (Wako). To minimize experimental deviation, CAT mRNA (Gibco) was added as an internal standard besides constant total RNA contents. The single stranded cDNA was amplified by PCR that was performed at 94°C for 1 minute followed by at 65°C for 3 minutes by repeating the reactions for 26 and 28 cycles. The primer pairs are 21mers and designed on the *sts* from *Pinus sylvestris* (Accession No. X60753 in GenBank). Deviation of the cDNA synthesis and PCR among the samples were compensated by the CAT internal standard. The band amplified was purified with a QIAquick spin column (Qiagen) and directly cycle-sequenced with FITC-labeled primer (Fig. 2) and SequiTherm DNA polymerase (AR Brown) to confirm as a *sts* by an automated DNA sequencer.

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                      1480        1490        1500        1510        1520
stsW1HcDNA      1451 CwCTGCCAAC ATTGAAAACT GTATGGTCGA GGCGTTCAGT CAGTwCAAAA    1500
sts(X60753)     1451 CTCTGCCAAC ATTGAAAACT GTATGGTCGA GGCGTTCAGT CAGTTCAAAA    1500
chs(X60754)     1451 CTCcAAgAAC ATaGAgAAga GTcTGGTtGA GGCcTTCcG- CAGTTCggAA    1500

                      1530        1540        1550        1560        1570
stsW1HcDNA      1501 TATCCGACTG GAACAAGTTG TTCTGGGTTG TTCATCCCGG AGGACGTGCC    1550
sts(X60753)     1501 TATCCGACTG GAACAAGTTG TTCTGGGTTG TTCATCCCGG AGGACGTGCC    1550
chs(X60754)     1501 TcTCgGACTG GAACcAGTTa TTCTGGaTcG caCATCCCGG AGGtCcTGCC    1550

                      1580        1590        1600        1610        1620
stsW1HcDNA      1551 ATCCTTGATC GGGTGGAGGC CAAGCTCAAT CTGGATCCCA CAAAACTGAT    1600
sts(X60753)     1551 ATCCTTGATC GGGTGGAGGC CAAGCTCAAT CTGGATCCCA CAAAACTGAT    1600
chs(X60754)     1551 ATtCTgGATC aGGTGGAGGC CAAGCTaAAT tTGGATCCCA agAAACTGAg    1600

                      1630        1640        1650        1660        1670
stsW1HcDNA      1601 ACCCACCAGG CACGTTATGA GCGAGTACGG AAACATGTCC AGTGCGTGCG    1650
sts(X60753)     1601 ACCCACCAGG CACGTTATGA GCGAGTACGG AAACATGTCC AGTGCGTGCG    1650
chs(X60754)     1601 tgCAACgAGG CAaGTacTGA GCGAcTAtGG AAACATGTCg AGcGCGTGCG    1650

                      1680        1690        1700        1710        1720
stsW1HcDNA      1651 TCCACTTCAT ATTGGATCAG AmnAGGAAGc ---------- ----******    1700
sts(X60753)     1651 TCCACTTCAT ATTGGATCAG ACGAGGAAGG CGTCTCTACA AAACGGATGT    1700
chs(X60754)     1651 TgCACTTCAT cTTGGAcgAG AtGAGGAAGt CcTCTaaAgA gAAaGGATGT    1700

                      1730        1740        1750
stsW1HcDNA      1701 ********** *****..... ..........    1750
sts(X60753)     1701 TCAACCACCG GAGAGGGATT GGAAATGGGA    1750
chs(X60754)     1701 TCcACCACCG GAGAGGGAcT GGAtggGGGA    1750
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Fig. 2. Multiple Sequence Alignment for chalcon synthase gene family in pine trees. stsW1HcDNA : a sequenced stilbene synthase cDNA from *Pinus densiflora*. sts (X60753) : a stilbene synthase gene from *Pinus sylvestris*, and the sequence was retrieved from GenBank (accession no. : X60753). chs (X60754) : a chalcone synthase gene from the same species. The sequence was from the same DNA data bank (accession no. : X60754). Astarisks show the posision where the backward primer locates. Small letters show mismatched bases with the sequence of X60753. The numbers show the base position from the 5' end of sts (X60753). The Gaps(–) in those sequences were inserted for a maximum alaignment.

3. Results and Discussion

The RT (reverse transcription)-PCR product amplified from the Japanese red pine, was ca.1.0 kbp in size, that is in good accordance with a *sts* expected (1036 bp) from the Scots

pine. Recently, the *sts* has recognized as a derivative from chalcone synthase genes[9], and both of the sequences and enzyme reactions are quite similar (Fig. 1). Thus, it has to demonstrate if the amplified fragment is real *sts*, although the fragment size is an expected one. The *sts* cDNA fragments from the Japanese red pine were partially sequenced (ca. 0.3 kbp) and aligned against those of *sts* and chalcone synthase gene from Scot pine (Fig. 2). The cDNA from the Japanese pine is 94% and 97% homologies in a *sts* from the Scots pine with amino acid and nucleotide sequences, respectively. On the other hand, it is 69% and 75% homologies in a chalcone synthase gene from Scots pine with amino acids and nucleotide sequences, respectively. Thus, the sequence obtained from the Japanese red pine clearly shows the one from *sts* but not from chalcone synthase gene.

Conifer species are not fully converged into a pure line comparing with vegetative crops, and are expected to contain genetic variations within a species. We selected two phenotypes observed in the Japanese pine seedlings. One has a reddish hypocotyle and the other was a pale one. The phenotypes under stress may respond differently. One of the stress applied was to uproot seedlings. The uprooted seedlings in the pine were diversely damaged after leaving them for 62 hours under moistened condition. There was a clear tendency that the reddish one is resistant while the pale one susceptible against the stress. The other stress was salicylate (SA) spray on the intact seedlings. It showed no apparent damage on the seedlings. However, the spray with or without SA induced *sts* expression that reached maximum level at 16 hours incubation. The pale seedlings with SA treatment showed higher *sts* level than ones with water control, while the expression level was almost same in the reddish ones. These observations suggest that individual seedlings may diverse responses to both of the stresses, i.e., uprooting and SA treatment. In conclusion, the susceptible seedlings to the uprooting show SA-sensitive *sts* expression, while resistant seedlings to the uprooting express *sts* with or without SA treatment.

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